COMPARATIVE STUDY OF *In vivo* AND *In vitro* METHODS IN DETERMINING PROTEIN DIGESTIBILITY OF FEEDS FOR BAGRID CATFISH, *Mystus nemurus*

by

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ABSTRAK

Kajian tentang kebolehpercayaan kaedah *in vitro* berbanding kaedah *in vivo* telah dijalankan untuk menentukan keterhadaman protein bagi diet kajian yang diformulasikan supaya mengandungi 35% protein dan 15% lipid bagi penggantian protein daripada serbuk ikan dengan serbuk kacang soya sebanyak 0 hingga 60% untuk ikan baung, *Mystus nemurus*. Sebagai sebahagian dari kajian ini, asai-asai biokimia menunjukkan aktiviti protease optimum pada pH 2.0 dan 9.0 pada bahagian perut dan usus masing-masing. Aktiviti protease yang paling tinggi ditunjukkan pada 2 dan 5 jam selepas pemberian makanan pada bahagian perut dan usus masing-masing. Kehadiran pepsin ditunjukkan di dalam perut manakala serine protease adalah lebih dominan di dalam usus, diikuti dengan tripsin, metalloprotease dan kimotripsin. Klasifikasi ekstrak daripada usus *M. nemurus* melalui kaedah SDS-PAGE dengan menggunakan perencat specific protease komersial menunjukkan kewujudan lapan protease beralkali dengan berat molekul di antara 8.4 dan 56.9 kDa. Sebelum formulasi diet kajian dilakukan, keterhadaman protein bahan mentah secara *in vitro* telah ditentukan dengan menggunakan kaedah pH stat, pH drop, asai spektrofotometri dan SDS-PAGE. Kaedah pH stat dan pH drop masing-masing menunjukkan turutan penurunan Darjah Hidrolisis (DH) dan Keterhadaman Protein Relatif (KPR) dengan serbuk ikan (SI) > serbuk kacang soya (SKS) > serbuk sotong (SS) > serbuk kacang soya mentah (SKSM) dengan menggunakan semua sistem enzim. Walau bagaimanapun, sistem Lazo 1- enzim melalui kaedah pH drop menunjukkan bahawa SI > SS > SKS > SKSM. Sistem Saterlee 4-enzim menunjukkan DH dan KPR tertinggi untuk semua bahan mentah diikuti dengan

menggunakan ekstrak enzim daripada usus *M. nemurus*, sistem Hsu 3-enzim dan Lazo 1-enzim. Asai spektrofotometri dan SDS-PAGE secara *in vitro* menunjukkan bahawa ekstrak enzim daripada usus *M. nemurus* direncat oleh SKSM pada tahap yang paling tinggi diikuti dengan SS, SKS dan SI. Zimogram daripada SDS-PAGE menunjukkan bahawa prainkubasi SKSM dengan ekstrak enzim daripada usus *M. nemurus* mengakibatkan pengurangan keamatan pada 3 jalur protease dan kehilangan 5 jalur protease. Secara keseluruhan, keterhadaman protein bagi diet kajian menggunakan kaedah *in vivo* menurun apabila kandungan SKS meningkat manakala peratus perencatan pula meningkat apabila kandungan SKS meningkat. Kadar penerimaan penggantian SKS oleh *M. nemurus* adalah sehingga 10% apabila ditentukan melalui kaedah *in vivo*. Kaedah pH stat dan pH drop menunjukkan nilai DH dan KPR yang paling tinggi apabila sistem Saterlee 4-enzim digunakan diikuti dengan ekstrak enzim daripada usus *M. nemurus*, sistem Hsu 3-enzim dan Lazo 1-enzim. Korelasi yang paling tinggi di antara kaedah *in vitro* berbanding kaedah *in vivo* ditunjukkan dengan menggunakan asai spektrofotometri diikuti dengan pH stat, SDS-PAGE dan pH drop walaupun tidak menunjukkan perbezaan secara signifikan di antara kaedah-kaedah *in vitro*.

COMPARTIVE STUDY OF *In vivo* AND *In vitro* METHODS IN DETERMINING PROTEIN DIGESTIBILITY OF FEEDS FOR BAGRID CATFSH, *Mystus nemurus*

ABSTRACT

The reliability study of the *in vitro* methods in relation to the *in vivo* method was conducted to assess the protein digestibility of experimental diets formulated at 35% protein and 15% lipid with 0 to 60% increasing protein substitution of fishmeal by soybean meal for bagrid catfish, *Mystus nemurus*. As a part of this study, biochemical assays showed optimum protease activity at pH 2.0 and 9.0 in the stomach and intestiness respectively. Protease activity of post feeding study in the stomach and intestines were highest at 2 and 5 hours accordingly. Pepsin was observed in the stomach while serine proteases were more dominant in the intestines, followed by trypsin, metalloproteases and chymotrypsin. Characterization of crude intestinal extract of *M. nemurus* via SDS-PAGE using commercial specific protease inhibitors revealed eight alkaline proteases with molecular weights between 8.4 and 56.9 kDa. Prior to formulation of experimental diets, *in vitro* protein digestibility of feedstuffs was assessed using pH stat, pH drop, spectrophotometric assay and SDS-PAGE methods. pH stat and pH drop methods revealed a decreasing order of respective Degree of Hydrolysis (DH) and Relative Protein Digestibility (RPD) with fishmeal (FM) > soybean meal (SBM) > squid meal (SM) > raw soybean meal (RSBM) using all enzyme systems. However, RPD for pH drop method using Lazo 1-enzyme system showed FM > SM > SBM > RSBM. Saterlee 4-enzyme system revealed the highest DH and RPD values for all feedstuffs followed by the crude intestinal enzyme extract of *M. nemurus*, Hsu 3-enzyme and Lazo 1-enzyme system. *In vitro* spectrophotometric assay and SDS-PAGE demonstrated that crude intestinal enzyme extracts of *M. nemurus* were inhibited

the highest by RSBM followed by SM, SBM and FM. Zymogram from SDS-PAGE showed that preincubation of RSBM with crude intestinal enzyme extract of *M. nemurus* resulted in 3 reduced and 5 disappeared protease bands. Overall, protein digestibility of experimental diets using *in vivo* and *in vitro* methods decreased as SBM protein substitution level increased while percentage of inhibition increased as SBM protein substitution level increased. The highest tolerance level of SBM protein substitution for *M. nemurus* was up to 10% which was determined using the *in vivo* method. pH stat and pH drop methods showed the highest DH and RPD using Saterlee 4-enzyme system followed by crude intestinal enzyme extract of *M. nemurus*, Hsu 3-enzyme and Lazo 1-enzyme system. The highest correlation of the *in vitro* methods with the *in vivo* method was obtained using spectrophotometric assay followed by pH stat, SDS-PAGE and pH drop although no significant differences were observed among the *in vitro* methods.

CHAPTER 1

INTRODUCTION

1.1 Current State of World Aquaculture

Aquaculture continues to play a very crucial role as a source of food for poverty alleviation in poor regions and also a revenue earner in many countries. The world production of aquaculture has increased immensely since the 1950s, from less than a million tonne to 59.4 million tonnes by 2004, valued at USD70.3 billion (FAO, 2006). China remains the largest contributor, with a production volume of 41.3 million tonnes or 69.6% followed by the rest of the Asia-Pacific region at 21.9%. From the total aquaculture production in 2004, marine fish culture accounted for 50.9%, consisted of mostly mollusks (42.9%) and aquatic plants (45.9%). Freshwater fish culture represented 45.4% of which more than 94% was from finfish production. On the other hand, brackish water culture contributed only 5.7% of the total aquaculture production (FAO, 2006). According to FAO (2006), an additional 40 million tonnes of aquatic food production is necessary to meet consumer requirements following the projected growth in human population for the next 20 years.

1.2 Introduction of Bagrid Catfish, *Mystus nemurus*

Bagrid catfish, *Mystus nemurus* is one of the most important freshwater fish in Southeast Asia, particularly Malaysia and Thailand is (Amornsakun *et al*., 1998; Leesa-Nga *et al*., 2000). The wild catch production of *M. nemurus* in dams of Peninsular Malaysia was reported to be 45.9 tonnes in 2004, mostly from Terengganu, Kelantan, Perak, Kedah and Johor (Anonymous, 2004). The price of *M. nemurus* in Malaysia can fetch up to RM10 to 15 per kilogram of fish in local markets, depending on season and location. The high demand of *M. nemurus* is due to its excellent taste, non-bony flesh and high content of protein, which has led more fish farmers to culture it. However, according to local farmers, the catch fisheries industry of *M. nemurus* is reported to be on the decline over the last few years and a proper culture technique of the fish is to be established in Malaysia.

Feeding of cultured *M. nemurus* is commonly performed using trash fish or available commercial feeds specifically formulated for *Oreochromis* sp. or *Clarias* sp. The dependence of fish farmers on the availability of feeds rather than suitability has triggered the necessity for further nutrition studies on *M. nemurus,* to formulate a suitable and cost effective feed that meets the nutrient requirements specifically designed for the mass production of this fish.

1.3 Issues Regarding the Present Study

The expansion of aquaculture to meet the rising demand for food supply also increases the demand for more feeds from aquaculture feed industry, especially for intensive fish culture. Global aquaculture feed production was 19.5 million tonnes in 2003 and was predicted to increase to 17.5 million tonnes by the end of 2010 (Hardy, 2006). In aquaculture feed industry, protein is one of the most expensive and important nutrient for fish growth. In view of this, studies on the quality and optimal requirements of protein for *M. nemurus* are important, to reduce feed costs. Moreover, feeds containing high level of protein do not necessarily indicate better fish growth but depends more on the bioavailability of the amino acids. The ability of fish to utilize proteins is related to the existence of different proteases in the digestive system which are responsible for hydrolyzing or breaking down the proteins into peptides and amino acids and eventually absorbed by the fish (Hepher, 1988).

However, there is insufficient available information of the activity of proteases in *M. nemurus*. Information on proteases could probably explain the differences in digestive capacities of protein of this fish because proteases are extremely specific towards their substrates. The identification of proteases present in the digestive system of *M. nemurus* and optimum conditions for their activities such as pH can be used to predict the suitable protein source for better feed utilization. Post feeding studies and rhythm of the protease activity can also be manipulated to improve feeding strategy for least cost total aquaculture production (Hashim, 1994).

The main protein source used in aquaculture feed industry is fishmeal. This is due to its superior amino acids profile, essential fatty acids content, high digestiblity and palatablity to fish (Peres *et al*., 2003). In 2006, over 46% of total fishmeal production was utilized for aquaculture feeds (Hardy, 2006). However, due to low production and

consistent increase in demand, the price of fishmeal rose to USD880 per tonne in 2006, the highest in history (Anonymous, 2006). This has forced the aquaculture feed manufacturers to use cheaper alternative protein sources as fishmeal protein replacement, to sustain the developing aquaculture production (Alexis and Nengas, 2001).

Various studies have been conducted, to evaluate the effects of partial or complete replacement of fishmeal protein with animal and/or plant protein sources. Generally, the animal protein replacement studies are usually conducted using fisheries by-catch and by-product meal (Li *et al*., 2004; Nwanna, 2003), meat meal (Stone *et al*., 2000; Williams, 2003), blood meal (Bureau *et al*., 1999), feather meal (Grazziotin *et al*., 2006; Bureau *et al*., 2000), bone meal (Zhou *et al*., 2004) and poultry by-products (Giri *et al*., 2000). However, the quality of animal protein including fishmeal is unstable and depending on species, temperature and processing methods (Bassompierre *et al*., 1997). Therefore, this has given rise for a pressing need to use plant protein sources instead, such as lupin meal (Farhangi and Carter, 2001; Glencross *et al*., 2003, 2006), pea meal (Borlongan *et al*., 2003), cottonseed meal (Cheng and Hardy, 2002; Lee, 2002; Lee *et al*., 2006), canola meal (Glencross *et al*., 2004), corn gluten meal (Zhou *et al*., 2004) and soybean meal (Sales and Britz, 2002; Chou *et al*., 2004; Gomez-Requeni *et al*., 2004).

The principal objective of the present study is to develop a nutritionally cost effective feed for mass production of *M. nemurus*. Therefore alternative plant protein sources would be evaluated to replace fishmeal protein. Several researchers have shown the potential of partially and totally replacing fishmeal protein with soybean meal protein in various species (Reigh and Ellis, 1992; Carter and Hauler, 2000; Laining *et al*., 2003; Chou *et al*., 2004). In the present study, soybean meal protein replacement

was selected due to its fairly balanced amino acids profile, cheaper price and unlimited supply. However, soybean is inferior to fishmeal in its content of certain amino acids; like methionine (Pillay and Kutty, 2005), cystine (Tibaldi and Tulli, 1999) and lysine (Bai *et al*., 2005) and contain high content of antinutritional factors compared to fishmeal (Francis *et al*., 2001; Peres *et al*., 2003). Therefore, studies on the inclusion levels of soybean meal protein in feeds for *M. nemurus* are also important.

One of the common methods to assess the degree of protein utilization of different protein sources in fish is through digestibility studies. Quantitative measurement of the nutritional efficiency of feedstuffs or feeds is commonly performed using the conventional *in vivo* method (Laining *et al*., 2003; Hernandez *et al*., 2007). However, several constraints like difficulties in faecal mater collection, nutrient leaching, high cost and maintenance of experimental fish, high labor costs and dependence of results on many environmental conditions (Ezquerra *et al*., 1998) have encouraged the development of a rapid and reliable *in vitro* method. This is to enable faster means of assessing the protein digestibility of feedstuffs or feeds, due to the increase in demand for the aquaculture industry (Lemos *et al*., 2000). The *in vitro* methods can also be used to rapidly evaluate the protein quality and the presence of protease inhibitors in selected protein sources before incorporating them in feed formulation.

In vitro digestibility has formerly been conducted on terrestrial animals using commercial proteases extracted from terrestrial animals and bacteria (Hsu *et al*., 1977; Saterlee *et al*., 1979). However, due to the differences in physiological properties of terrestrial animals compared to fish, the *in vitro* methods using terrestrial animals and bacterial proteases are not suitable for determining the protein digestibility of fish (Glass *et al*., 1989). Therefore, studies on proteases extracted from the experimental fish

are also necessary to compare the protein digestibility values (Dong *et al*., 1993; Dimes and Haard, 1994; Divakaran *et al*., 2004).

The general objectives of this study were to determine the optimum pH for protease activity in the stomach and intestine of *M. nemurus*. Results obtained would be used to assess the protease activity during food ingestion at different post feeding hours. Inhibition studies using commercial specific protease inhibitors for both stomach and intestine and the identification of major alkaline proteases in the intestine of *M. nemurus* via SDS-PAGE would also be assessed.

Protein digestibility of selected feedstuffs would be determined using only the *in vitro* methods while protein digestibility of experimental diets would be determined using both the *in vivo* and the *in vitro* methods via pH stat, pH drop, spectrophotometric assay and SDS-PAGE. Results of the protein digestibility of experimental diets from the *in vivo* method would then be correlated with the *in vitro* methods to determine the most suitable method to measure protein digestibility for *M. nemurus*.

CHAPTER 2

LITERATURE REVIEW

2.1 Morphology of Bagrid Catfish, *Mystus nemurus*

Mystus nemurus is categorized into the family of Bagridae and is known locally as baung, tropical catfish (Khan *et al.*, 1996), green catfish (Amornsakun *et al*., 1997, 1998) or bagrid catfish (Ng *et al*., 2000; Muchlisin *et al*., 2006). It can be found in freshwater, estuarine and upstream of reservoirs. *M. nemurus* can be classified as omnivorous species but have the tendency to feed like carnivorous-scavengers (Amornsakun *et al*., 1998). The natural diet of *M. nemurus* consists of fish, shrimps, insects, benthic crustaceans and detritus (Amornsakun *et al*., 1998).

Morphologically, *M. nemurus* has a wide and flattened head with compressed-like posterior body part. Upper jaw is longer than the lower (Mohsin and Ambak, 1992) and it has a big subterminal mouth characteristic (Taki, 1974). *M. nemurus* has no scale and the skin at the dorsal part of the body is grey but brighter at the ventral. There is one thorn with serrated appearance at the dorsal fin and one at each pectoral fin (Kottelat *et al*., 1993). The adipose fin is shorter than the dorsal fin but nearly the same length as the anal fin (Taki, 1974). It has dicercal caudal fin and four sets of barbells; nasal, maxillary, mandibulary and mental barbells. The morphology of *M. nemurus* is presented in Figure 2.1.

Figure 2.1 Bagrid Catfish, *Mystus nemurus*

2.2 Fishmeal as Protein Source in Aquaculture Feed

Fishmeal is the main contributor of protein source in aquaculture feeds due to its balanced amino acids profile, essential fatty acids, minerals, low carbohydrates, proper in digestion and highly palatable (Peres *et al*., 2003). Fishmeal is commercially made from the whole fish like chapelin, herring, anchovy or menhaden white fish including the bones and offal after lipid extraction. The composition of fishmeal depends on the types of raw material processing methods. In herring fishmeal, the nutrient composition comprise of approximately 8% moisture, 71% protein, 9% lipid and 12% ash, while lower nutrient composition was found in whitefish meal which constitute of 8% moisture, 66% protein, 5% lipid and 21% ash (Fox *et al*., 2004).

Although fishmeal is highly digestible compared to protein from plant sources, the main issue with fishmeal usage is its inconsistent quality which is dependent on species, time of capture, methods of transportation, state of fish body, temperature and types of processing method (Bassompierre *et al*., 1997). In 2005, productions of fishmeal in countries like Peru, Chile, Iceland, Denmark and Norway declined by 8% from the overall production (Anonymous, 2006). The production of fishmeal was unable to match the increasing demand, hence the resulting increase in fishmeal price. This has pressured the nutritionists and feed manufacturers to search for alternative protein sources which are nutritionally available, cheap and stable in supply such as the soybean meal.

2.3 Soybean Meal as Protein Replacement

Soybean (*Glycine max*) is regarded as a nutritive and important plant protein that has been extensively used in fish feed (Chou *et al*., 2004). It comprises of about 56% of the world oilseed crops (Kerley and Allee, 2003) with the United States being the

biggest producer followed by Brazil and the European Union (Anonymous, 2004). Soybean meal is derived from a by-product soybean seed after lipid extraction which contains about 47 to 50% protein, 1% lipid, 40% carbohydrate and 5 to 6% ash (FAO, 1980). Soybean protein has favorable amino acid profile and the low priced availability is theoretically limitless (Chou *et al*., 2004). This has encouraged the substitution of fishmeal protein to soybean products like full-fat soy, soybean meal and soy protein concentrate which can reduce the cost of aquaculture feeds. The general composition of the soybean products according to Alexis and Nengas (2001) is presented in Table 2.1.

Table 2.1 General composition of soybean products (%).

Types of Soybean	**Moisture**	**Protein**	**Lipid**	**Fiber**	**Ash**
Full-Fat Soybean Meal (FSBM)	10.0	38.0	18.0	5.0	4.1
Extracted Soybean Meal (SBM)	11.0	45.0	1.2	6.1	6.1
Soy Protein Concentrate (SPC)	8.0	84.0	0.5	0.1	3.5

Compared to fishmeal, different soybean products have varying nutrient composition, low in essential amino acids, energy, minerals and digestibility, less palatable and contain antinutritional factors which affect the fish diversely (Peres *et al*., 2003). Thus, comprehensive research on the quality, acceptability and inclusion levels of soybean meal have been done in various fish species to provide nutritionally balanced and least cost feed. However, insufficient information is available on the use of soybean meal in the diets of *M. nemurus*. The protein replacement study iinformative to minimize feed cost while maintaining the nutritive value necessary for this fish.

2.3.1 Inclusion Levels of Soybean Meal

Different fish display various degree of acceptability towards soybean products. Although some fish may have comparable taxonomical and physiological characteristics, the ability to digest the same ingredients is significantly different due to the specific capabilities in digestion among species (Glencross *et al*., 2004).

Full-fat soybean meal (FSBM) is produced from the treated whole seed and is usually accepted at a lower inclusion level by fish. In carnivorous *Gadus morhua*, the use of FSBM revealed a low acceptance of 14% (Albrektsen *et al*., 2006). Boonyaratpalin *et al*. (1998) found that diets containing extruded FSBM, steamed FSBM or soaked raw FSBM diets showed significantly lower growth parameters in *Lates calcarifer* compared to diet with fishmeal protein. Opstvedt *et al*. (2003) stated that in *Salmo salar*, the apparent protein digestibility (APD) of FSBM diet is lower than the fishmeal diet with the values of 76.9 and 89.3% respectively. Nengas *et al*. (1995) found lower APD of FSBM (75.7%) in *Sparus aurata* compared to SBM diet (90.9%). The comparatively lower inclusion level and APD of FSBM in fish feed is due to lower protein, energy and mineral content, high in fiber content and contains high level of antinutritional factors which inhibit the protease activity, hence reducing growth and nutrient digestibility (Alexis and Nengas, 2001). Therefore, extracted soybean meal is widely used to study the replacement of fishmeal protein in fish feed.

Extracted soybean meal (SBM) is obtained as a by-product after lipid extraction and contains higher protein but lower lipid content compared to FSBM. SBM is widely used to study the replacement of fishmeal protein in fish feed due to the high APD values in various species. More than 90% of the APD value was obtained in *Rachycentron canadium* fed diets with defatted soybean meal (Zhou *et al*., 2004). The APD of 79.95% were observed in hybrid *Morone saxatilis* x *M. chrysops* with 30%

SBM replacement (Sullivan and Reigh, 1995). Carter and Hauler (2000) revealed that the APD of 33% SBM replacement diet for *Salmo salar* was significantly higher than the commercial and control diet with similar protein content.

The high acceptance level of SBM was also shown in other cultured fish. *Ictalurus furcatus* demonstrated high acceptance level of 69% SBM replacement in its diet (Webster *et al*., 1992). In a study done by Reigh and Ellis (1992), they revealed that the carnivorous *Sciaenops ocellatus* can tolerate the increment of 50% SBM protein which did not reduce the growth and protein utilization significantly. Comparable result was also seen in *Paralichthys olivaceus* (Kikuchi, 1999) and *Rachycentron canadum* (Chou *et al*., 2004) with the acceptance level of SBM inclusion approximately at 45 and 40%, respectively. In *Sparus aurata*, no significant difference in growth was observed when fishmeal was replaced by SBM protein up to 30% (Robaina *et al*., 1995).

On the other hand, lower APD and dry matter digestibility was reported in *Cromileptes altivelis* fed 30% SBM replacement with the values of 67.2 and 54.8%, respectively (Laining *et al*., 2003), similar to *Sciaenops ocellatus* (63.6%) studied by Gaylord and Gatlin (1996). McGoogan and Reigh (1996) found lower digestibility (37.8%) with SBM replacement in *Sciaenops ocellatus*. A very low acceptance level of defatted SBM (20%) was observed in *Salmo salar* but at 40% replacement, growth was reduced (Olli *et al*., 1995). Similar acceptability of 20% SBM replacement was demonstrated in *Nibea miichthioides* without significant differences in final body weight (Wang *et al*., 2006). Bonaldo *et al*. (2006) stated that no significant growth performance, nutrient utilization and gut histology was observed in *Solea aegyptiaca* fed 30% SBM inclusion. For carnivorous fish like *Oncorhynchus mykiss*, even a low level of 25% fishmeal replacement by SBM decrease the growth, feed efficiency and protein retention (Kaushik *et al*., 1995).

High feed intake of SBM was also seen in certain fish. A study done by Wang *et al*. (2006) showed that among six diets with replacement of 0, 20, 40, 60, 80 and 100% SBM, the highest feed intake was shown in fish fed the 100% SBM diet although poor growth was observed with increased SBM substitution. Hernandez *et al*. (2007) also found that feed intake rose in *Diplodus puntazzo* SBM content higher than 40 percent but decreased in final weight with increased SBM. The increased of feed intake with high SBM content but resulted in low growth may probably indicate the lower amount of nutrient bioavailability of the SBM diet where the fish tend to consume more feed to meet the nutritional requirement. Although SBM diet is better digested by fish compared to FSBM diet, there are still several fish species which have low tolerance of SBM protein. In order to overcome this problem, studies were also conducted to use more purified soy product in fish feed which is claimed to be highly digestible and contain reduced antinutritional factors like soy protein concentrate (Storebakken *et al*., 1998a).

Soy protein concentrate (SPC) is obtained through aqueous or methanol extraction of soybean protein to reduce non starch polysaccharide, fiber and saponin content of soybean meal which improve its functional properties (Drew *et al*., 2007). Growth and feed intake in salmonid diets improved when SPC was incorporated into its diet (Kaushik *et al*., 1995). Hansen *et al*. (2007) also showed higher APD for *Gadus morhua* fed diet with 100% plant protein replacement containing SPC, SBM and wheat gluten compared to fishmeal diet. However, Day and Gonzalez (2000) showed lower acceptance level of SPC in the diet for *Scophthalmus maximus* (up to 25%). Although the limitation of the essential amino acids in soybean such as lysine, threonine and methionine have been improved in the SPC, Gatlin *et al*. (2007) stated that the high

processing costs to produce the SPC is uneconomical for large scale aquaculture feed industries.

All the different types of soybean products incorporated into the experimental diets exhibit varying effects on the fish which eventually affects growth. However, among the main factors in soybean products which are reported to reduce the protein digestibility in fish are the presence of trypsin inhibitors, deficiency in amino acid contents and the existence of the indigestible non starch polysaccharides and oligosaccharides.

2.3.2 Trypsin Inhibitors

Trypsin inhibitors exist mostly in legume seeds and cereal. It is a single polypeptide chain, crosslinked by two disulfide bridges which contain 181 amino acid residues. Trypsin inhibitors have molecular weight of 20.1 kDa (Kunitz, 1947) and mainly inhibits trypsin, chymotrypsin (at lower level) and other proteases with parallel mechanism as trypsin by forming insoluble complexes with the proteases (Dimes *et al*., 1994a; Peres *et al*., 2003) and making them unavailable for protein hydrolysis. However, trypsin inhibitors will not inhibit acid proteases and metalloproteases (Kunitz, 1947).

Trypsin inhibitors show significant effects on fish proteases compared to mammals (Alexis and Nengas, 2001) but can still be tolerated by fish at lower than 5 mg trypsin inhibitors per gram (mg/g) of meal (Francis *et al*., 2001). However, Wilson and Poe (1985) found that the growth for *Ictalurus punctatus* was affected when fed with SBM diets which contain higher than 2.2 mg of trypsin inhibitors per gram diet. Wee and Shu (1989) found that trypsin inhibitors at 1.6 mg/g diet reduced the growth of

Oreochromis niloticus but not at 0.6 mg/g diet. *Oncorhynchus mykiss* also showed high sensitivity towards trypsin inhibitors (Krogdahl *et al*., 1994).

The high tolerance of trypsin inhibitors in certain fish is probably due to the ability of the fish to enhance the production of trypsin in the pancreas (Alexis and Nengas, 2001; Francis *et al*., 2001) which increase protein hydrolysis (Krogdahl *et al*., 1994). Olli *et al*. (1994) stated that higher trypsin was produced when diets for *Salmo salar* contained 4.8 mg trypsin inhibitors per gram diet. In FSBM, trypsin inhibitors are usually present unless it is reduced through extrusion or expelling processes (Cheng and Hardy, 2003).

The effects of trypsin inhibitors are commonly reduced by heat treatments. Heat treatment denatures the indigenous protein structure and the trypsin inhibitors, hence improving the nutritive value of SBM (Herkelman *et al*., 1992). Peres *et al*. (2003) reported that most of the trypsin inhibitors present in SBM were destroyed if heated at 130ºC for 40 minutes. Similar result was obtained by Haard *et al*. (1996) where soybean trypsin inhibitors activity were reduced by 99% when SBM was heated at 121ºC for 20 minutes before diet formulation (Haard *et al*., 1996). Grabner and Hofer (1985) reduced trypsin inhibitors in SBM by heat treatment at 140ºC for 30 minutes. When heating of SBM in fish diet is extended to 40 minutes, the feed intake was significantly lowered but showed higher feeding efficiency than the unheated SBM diet (Peres *et al*., 2003).

Although heat treatment is one of the methods used to reduce trypsin inhibitors in SBM, overheating can degrade essential amino acids like lysine and methionine (van den Ingh, 1991; Qin *et al*., 1998; Ljokel *et al*., 2000) arginine, cysteine, serine and threonine (Sorensen *et al*., 2002) thus affecting protein utilization in fish for growth. Heating of feed ingredients at 100 to 125ºC can slightly reduce protein digestibility and total amino acids, but the entire amino acids digestibility decreased at 150ºC or beyond

(Sorensen *et al*., 2002). Hence, heating period for SBM should be carefully considered to prevent the Maillard reaction because there is a slight margin between inactivating the antinutritional factors as a result of inadequate heating or destroying essential nutrients due to overheating (Arndt *et al*., 1999; Sorensen *et al*., 2002). Heat processing of SBM through extrusion or expelling practice can also destroy trypsin inhibitors and antinutritional factors in fish (Cheng and Hardy, 2003; Samocha *et al*., 2004; Aslaksen *et al*., 2007). Nevertheless, heating is ineffective in reducing heat stable inhibitors like Kunitz and Bowman-Birk (Francis *et al*., 2001).

Besides heating, trypsin inhibitors can also be removed by bioprocessing of SBM (to concentrate protein and remove antinutritional factors). According to Refstie *et al*. (2006), bioprocessed SBM contain reduced trypsin inhibitors (2.3 mg/g of protein) compared to untreated SBM (4.5 mg/g of protein). Bioprocessed SBM improved dietary value for *Salmo salar* as reported by Refstie *et al*. (1998, 2005). However, Refstie *et al*. (2006) concluded that no significant difference was observed in feed efficiency of *Gadus morhua* fed SBM and bioprocessed SBM which suggest the high tolerance of SBM in *Gadus morhua*.

2.3.3 Amino Acids Deficiency

Soybean meal (SBM) contains fairly balanced amino acids profile with high phenylalanine and arginine (Hertrampf and Piedad-Pascual, 2000) but deficient in methionine (Tibaldi and Tulli, 1999; Pillay and Kutty, 2005), cystine (Tibaldi and Tulli, 1999) and lysine (Bai *et al*., 2005; Pillay and Kutty, 2005). Several researchers found that deficiency in amino acids content affects growth. Although SBM is deficient in certain amino acids content, the important factor in formulating quality feed is the absorption efficiency of the protein ingested by the fish that is used for growth. Two

diets which contain the same amount of amino acids might have significant differences in absorption capabilities. Therefore, the bioavailability of a nutrient affects the fish growth and digestibility more significantly compared to the amount of nutrient ingested by the fish.

The insufficient amino acids can be supplemented in the SBM diets depending on the amino acid requirement of the fish which improves the protein utilization. The amino acid supplementation can also result in higher inclusion level of SBM protein in fish diets hence reducing feed cost due to lower fishmeal usage. Bai *et al*. (2005) stated that the addition of methionine and lysine increased the acceptability level of SBM inclusion up to 30% without affecting growth compared to only 20% SBM replacement without these amino acids supplementation. Studies by Lim *et al*. (2004) showed that *Sebastes schlegeli* which was fed with diets containing dehulled SBM gained weight significantly with the addition of amino acids. Sveier *et al*. (2001) found that *Salmo salar* was able to accept 75% of SPC in its diet with methionine addition. In hybrid *Morone saxatilis* x *M. chrysops*, the supplementation of methionine demonstrated high protein utilization with increased in SBM inclusion by up to 75% (Gallagher, 1994). Increased growth was shown in *Homarus americana* when arginine, leucine, methionine and tryptophan were supplemented in the diet with 50% SBM substitution to meet the amino acids requirement (Floreto *et al*., 2000).

Other studies did not show any significant difference in growth or protein utilization despite the amino acid supplementation into the SBM diets. Tibaldi *et al*. (2006) found that methionine supplementation did not affect growth performance of *Dicentrarchus labrax* when fishmeal was replaced with 25% of dehulled solvent-extracted soybean meal, 50% enzyme treated soybean meal, or 60% of both soybean products inclusion at a ratio of 1:1. Despite the low methionine content in SPC, the

growth of *Solea senegalensis* post larvae was not reduced (Aragao *et al*., 2003). This is probably due to the sufficient amino acids required by these fish despite the low content of amino acids in the SBM diets. On the other hand, the unavailability of the supplemented amino acids due to the inefficient absorption capabilities of the fish can also result in insignificant difference in fish growth compared to diets without amino acids supplementation. Similarly, the low content of methionine in SBM, did not affect the protein utilization in *Solea aegyptiaca* fed with 30% SBM replacement diet (Bonaldo *et al*., 2006). Therefore, formulating efficient feed does not necessary requires high protein content, but to meet the amino acid requirement and the absorption capabilities are more important.

Besides incorporating deficient amino acids in fish diets, combination of protein sources was reported to improve the amino acids profile. For instance, Albrektsen, *et al*. (2006) revealed that 50% fishmeal protein replacement by corn gluten meal and FSBM at a ratio of 2:1 did not reduce feed intake, growth and protein digestibility in *Gadus morhua*. Hansen *et al*. (2007) showed no significant reduction of APD and dry matter digestibility when fishmeal diets were totally replaced with a combination of SBM, SPC and wheat gluten meal with crystalline methionine and lysine supplementation. Amaya *et al.* (2007) stated that *Litopenaeus vannamei* showed excellent growth performance when fishmeal was replaced by the combination of solvent extracted SBM, corn gluten meal and corn fermented soluble with 1% squid meal. Muzinic *et al*. (2004) found that total replacement of fishmeal with a combination of SBM and brewer's grains with yeast did not affect the growth of *Cherax quadricarinatus*. The reduction of fishmeal protein used in aquaculture feeds can minimize the production cost and increase aquaculture profits. The comparison of the amino acid profile of fishmeal and soybean meal as reported by Alexis and Nengas (2001) is presented in Table 2.2.

Table 2.2 Amino acids profile of fishmeal and soybean meal.

Amino Acids	Fishmeal	Soybean Meal
Arginine	6.40	7.75
Lysine	7.50	6.35
Histidine	2.40	2.65
Isoleucine	4.50	5.10
Leucine	7.40	7.45
Valine	5.10	4.90
Methionine + Cystine	4.00	2.60
Phenylalanine	4.20	5.10
Threonine	4.00	4.05
Tyrosine	3.20	3.35
Tryptophan	1.10	1.20

2.3.4 Non Starch Polysaccharides and Oligosaccharides

Non starch polysaccharides (NSP) and oligosaccharides exist in grain legumes such as soybean meal (Storebakken *et al*., 1998a; Francis *et al*., 2001). NSP which is characterized into soluble and insoluble form is commonly used as feed binders, thickeners and gelling agents due to their ability to bind with water molecules (Kraugerud *et al*., 2007) to form gum-like compound (guar gum). However, the soluble NSP which consists of one third of the total NSP (Forde-Skjaervik *et al*., 2006) can result in high intestinal and faecal water content. This resulted in reduced protein digestibility in fish (Gatlin *et al*., 2007). The intestinal guar gum increases digesta viscosity which reduces the movement of intestinal content and distribution of

proteases, thus decreasing protein digestibility (Storebakken *et al*., 1998a; Francis *et al*., 2001).

In a study by Leehouwers *et al*. (2006), increased level of soluble NSP guar gum at 4% and 8% significantly decreased the APD in *Clarias gariepinus*. Amirkolaie *et al*. (2005) found that guar gum (soluble NSP) inclusion in the diets of *Oreochromis niloticus* significantly increased digesta viscosity and reduced growth and APD compared to diets with cellulose (insoluble NSP) inclusion.

Cellulose is less digestible in fish due to the lack of cellulase in the digestive system. However, Chakrabarti *et al*. (1995) found cellulase activity in several fish but it remains unclear whether the cellulase is endogenous to the fish or originated from microbes (Hansen and Storebakken, 2007). Studies by Francis *et al*. (2001) showed significant effects of cellulose content on protein digestibility in fish. Jantrarotai (1994) demonstrated poor performance of hybrid *Clarias* catfish when fed with diets containing 18% cellulose.

In other studies, dietary cellulose did not influence or have minor effects of APD in fish. Dias *et al*. (1998) and Aslaksen *et al*. (2007) found that cellulose inclusion up to 20 and 11% in *Dicentratchus labrax* and *Salmo salar*, respectively did not influence the APD of the fish. Hansen and Storebakken (2007) also demonstrated that 15% cellulose inclusion showed no reduced APD in *Oncorhynchus mykiss*. Amirkolaie *et al*. (2007) stated that less than 8% of cellulose content did not influence or have minor effects on APD in *Oreochromis* sp. Therefore, cellulose can still be used in nutritional studies of fish as feed filler at lower level (Hansen and Storebakken, 2007).

Another type of carbohydrate that was reported to decrease APD in fish is the oligosaccharides. Oligosaccharides exist in soybean meal at 15% but lower in soy protein concentrate (3%) (Gatlin *et al*., 2007). Oligosaccharides can reduce the amino

acid digestibility of fish, affect digestion of other nutrients and its own protein content (Glencross *et al*., 2003). Similar to NSP, oligosaccharides can also increase the viscosity of the intestinal content and decrease the nutrient absorption thus affecting growth performance in fish (Kraugerud *et al*., 2007).

Therefore, removal of oligosaccharides from the SBM by fermentation has been practiced to improve the amino acids digestibility (Refstie *et al*., 1998; Glencross *et al*., 2003) and enhance the absorption of nutrient for fish growth (Gatlin *et al*., 2007). Refstie *et al*. (1998) stated that growth performance and digestibility were higher in *Salmo salar* fed diets with reduced content of oligosaccharides and antinutritional factors (RO-SBM) compared to extracted SBM diets.

However, oligosaccharides content in SBM did not show reduced nutrient digestibility or growth in *Oncorhynchus mykiss* (Rumsey *et al*., 1993; Sanz *et al*., 1994; Kaushik *et al*., 1995), *Sarotherodon mossambicus* (Jackson *et al*., 1982) and *Cyprinus carpio* (Ufodike and Matty, 1983). Due to different effects of soybean products, studies on the substitution level of fishmeal with soybean protein should be carried out to estimate the protein digestibility of fish before formulating efficient feed.

2.4 Methods of Protein Digestibility

Protein digestibility involves a quantitative measurement of total protein in the diet ingested by the targeted species that is not recovered in the faeces. High content of protein and balanced amino acids profile of feedstuff or diet do not represent excellent digestibility if the protein or amino acids are not well digested and absorbed by the fish. In intensive farming, the nutrient requirements of a fish are exclusively provided by the aquaculturists as formulated diets. Therefore, the information of the protein bioavailability of selected feedstuffs or diets is crucial to produce effective and digestible feed. One of the methods is done by digestibility measurement.

Digestibility values depend on several factors such as the types and physical characteristics of the feedstuffs or diets, the varying experimental fish species and the physiological development stage of the fish (De Silva and Anderson, 1995). Different techniques of digestibility determination such as the use of dietary markers and the faecal matter collection can also affect the protein digestibility values. Conventionally, the *in vivo* method is used to measure the apparent protein and dry matter digestibility by quantifying the difference between the nutrient and marker contents in the feed with the nutrient and marker contents in the faeces using this formula:

Apparent Nutrient Digestibility =

$$100 - 100 \frac{[(\%\ \text{marker in the feed})\ (\%\ \text{nutrient in the faeces})]}{[(\%\ \text{marker in the faeces})\ (\%\ \text{nutrient in the feed})]}$$

Higher nutrient and lower marker content in the faeces compared to the diet result in lower nutrient digestibility while lower nutrient and higher marker content in the faeces compared to the diet demonstrate high nutrient digestibility.

2.4.1 *In vivo* Digestibility

In the *in vivo* digestibility method, dietary marker is often included into the formulated diet at 0.5 to 1.0% to measure nutrient digestibility. The marker should not interfere with the digestive metabolism of the fish, non-toxic, cannot be absorbed through the lumen and should move at a similar rate as feed material along the intestine (Smith and Tabrett, 2004). The amount of marker in the diet and faeces remains constant during the experiment and the ingested marker will appear in the faeces at the same amount. However, digestibility values can be affected in certain species due to slight assimilation of the marker and dissimilar movement of external markers compared to food materials (De Silva and Anderson, 1995).

Various markers have been done to measure nutrient digestibility such as hydrolysis resistant ash (HRA) or acid insoluble ash (AIA) (Sales and Britz, 2001; Goddard and Mc Lean, 2001), hydrolysis resistant organic matter (HROM), crude fiber, silica, microtracer F-Ni (Kabir *et al*., 1998), 32P (insoluble ammonium molybdate), titanium IV oxide (Richter, 2003) celite polyethylene, yttrium (Davies and Gouveia, 2006) and chromic oxide (Cr_2O_3) (Montano-Vargas, 2002). In this study, chromic oxide (Cr_2O_3) is used to measure the apparent protein and dry matter digestibility of the experimental diets in *M. nemurus*.

Different methods of faecal matter collection can also affect digestibility values. The common methods which require the collection of faecal matter after defecation by fish are netting, Guelph system (Eusebio *et al*., 2004; Vandenberg and De La Noüe, 2001) and settlement techniques (Booth *et al*., 2005; Glencross *et al*., 2007). However, leaching of nutrient and marker from the feed and faeces prior to faecal matter collection occurs and this can erroneously affect the estimation of APD values. Leaching is affected by the physical stability of the faeces, the length of time of faeces

in contact with water, temperature, water flow rate and the level of disturbance of feaces like siphoning and pipetting prior to faecal matter collection (Allan *et al*., 1999). Hajen *et al*. (1993a) found that nutrient leaching in the faeces left in the water for 18 hours are higher compared to the faeces left in the water for 6 hours in *Oncorhynchus tshawytscha*. This showed that longer period of faeces remained in the water resulted in higher degree of nutrient leaching and overestimation of digestibility value. The same nutrient leaching effect was also shown by Henken *et al*. (1985) and Jones and De Silva (1997).

There were other preferred methods of faecal matter collection in order to avoid nutrient leaching. The faecal matter is obtained from the intestine by dissection (Peres *et al*., 2003; Storebakken *et al*., 1998b), anal suction (Percival *et al*., 2001) and stripping (Glencross *et al*., 2006; Cheng and Hardy, 2003). The efficacy of these methods varies depending on species. Førde-Skjærvik *et al*. (2006) found that faecal matter collection using dissection method produced higher APD in *Gadus morhua* compared to stripping method. Lower digestibility value obtained using stripping method in this study is probably due to the higher number of fish needed to collect enough samples for digestibility determination. The stripping method used might coincidently contaminate the faeces sample with the mucus and urine from the fish and inaccurately lowers the digestibility values. Stripping can also resulted in high mortality rate and lost of scales (Dong *et al*., 1993) and not suitable for highly stressed fish like *Symphysodon aequifasciata* (Chong, 2000). Allan *et al*. (1999) stated that stripping method is not suitable as faecal matter collection in juvenile *Bidyanus bidyanus* and dissection method produced lower digestibility values compared to settlement technique.

On the other hand, Storebakken *et al*. (1998b) found no significant difference of APD in juvenile (131g) *Salmo salar* using both dissection and stripping methods but